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Award Number: DAMD17-02-1-0123

TITLE: Methylation Mediated Repression of Selected Genes in Prostate Cancer

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CONTRACTING ORGANIZATION: Department of Veterans Affairs
Washington, DC 20420

REPORT DATE: April 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2003	Annual (1 Apr 02 - 31 Mar 03)

4. TITLE AND SUBTITLE
Methylation Mediated Repression of Selected Genes in Prostate Cancer

5. FUNDING NUMBERS
DAMD17-02-1-0123

6. AUTHOR(S) :
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The present project is focused on understanding the exact mechanism by which methylation silences gene expression in prostate cancer cell lines. Certain proteins bind preferentially to methylated DNA and these proteins have been shown to repress gene expression. In order to determine which of these protein or proteins interact with methylated genes inside the cells, we plan to use using chromatin immunoprecipitation assay. Two important requirements for this assay include an optimal sonication of the fixed chromatin (most of the sonicated fragments should be around 500bp) and quantitative PCR assay for the gene under study. We have determined the linear PCR conditions for the proposed genes as well as the optimal fixation and sonication conditions for the prostate cancer cell lines. We have performed chromatin immunoprecipitation assay for the GSTP1 and AR promoters in LNCaP prostate cancer cell lines. As compared to the *AR* promoter, the *GSTP1* promoter was enriched in deacetylated histones H3 and H4. These results are consistent with the recruitment of histone deacetylase containing complexes by methylated DNA, resulting in a localized deacetylation.

14. SUBJECT TERMS:
methylation, histone deacetylation, gene repression, prostate cancer, selenium demethylation, chromatin immunoprecipitation, in vivo DNA-protein interaction

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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The progress report covers the period April 1, 2002 - March 31, 2003. However, the distribution of funds was stopped effective October 1, 2002 pending the transfer of Prinicipal Investigator to University of Miami. Therefore, the report here describes the work performed during the first 6 months of the funding of the project.

Introduction:

Prostate cancer is the most frequently diagnosed neoplasm and the second leading cause of cancer mortality in men after lung cancer (5). The mechanisms underlying prostate carcinogenesis, tumor progression, and metastatic dissemination remain undetermined. It has been proposed that changes in gene expression through an epigenetic mechanism may be the main force in prostate cancer progression (9). A number of genes have been shown to be aberrantly methylated in prostate cancer cell lines and human prostate cancer tissue specimens (2, 4, 6-8). Direct binding of specific transcriptional repressors to methylated DNA appears to be a major mechanism of transcriptional repression (1, 10). Four methyl-CpG-binding proteins (MBD1, MBD2, MBD3, and MeCP2) have been implicated in transcriptional repression (1) through an interaction with a histone deacetylase complex. Our hypothesis is that methylation represses expression of the proposed genes by the binding of the methylated promoter region by one or more of the methyl-CpG-binding proteins, which interact with a histone deacetylase complex. Numerous studies in animal models and recent studies in humans have demonstrated cancer chemopreventive effects of selenium (3). Our hypothesis is that the mechanism of action of selenium compounds involves demethylation and activation of aberrantly methylated genes in prostate cancer.

Body

Task 1. To determine which methyl-CpG-binding proteins bind to methylated promoters of selected aberrantly methylated genes in prostate cancer cells in vivo (months 1 –12):

- Determine linear PCR conditions for the proposed genes (months 1-2)
- Determine optimal fixation and sonication conditions for the prostate cancer cell lines (months 3-6).
- Perform chromatin immunoprecipitation assay (months 7-12).

Chromatin Immunoprecipitation (CHIP) Assay

Formaldehyde cross-linking can be used to rapidly fix in vivo protein-protein and protein-DNA complexes to conserve and analyze native structures. Antibody is used to immunoprecipitate a protein of interest from the whole-cell extract (11, 12). This results in the co-precipitation of any DNA that was directly or indirectly cross-linked to that protein. Reversal of the protein-DNA crosslinks allows isolation and purification of the co-precipitated DNA. A PCR amplification using co-precipitated DNA as template is performed to test whether certain chromosomal sequences have been enriched relative to others. Enrichment indicates DNA sequences that are associated with the protein of interest in vivo.

Two important requirements for this assay are -

1. Optimal sonication of the fixed chromatin - most of the sonicated fragments should be around 500bp.

2. Linear PCR conditions for the gene under study

A. Determine linear PCR conditions for the proposed genes (months 1-2)

In the original proposal, we have mentioned that PCR amplification will be carried out with ^{32}P-dCTP in the reaction mixture. PCR products will be separated on a 5% nondenaturing polyacrylamide gel, and bands will be quantitated by phosphorimager analysis. Since a real-time PCR machine at the LSU Health Science Center, Shreveport, LA, became available in the core facility, we have decided to use real time PCR for template quantification. Real-time PCR quantitates the initial amount of the template most specifically, sensitively and reproducibly, and is a preferable alternative to other forms of quantitative PCR, which detects the amount of final amplified product

Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e., in real time) as opposed to the endpoint detection by conventional quantitative PCR methods. Real-time PCR quantitation eliminates post-PCR processing of PCR products which reduces the chances of carryover contamination and removes post-PCR processing as a potential source of error. In comparison to conventional PCR, real-time PCR also offers a much wider dynamic range of up to 10^7-fold (compared to 1000-fold in conventional PCR). This means that a wide range of ratios of target and normalizer can be assayed with equal sensitivity and specificity. It follows that the broader the dynamic range, the more accurate the quantitation.

The real-time PCR system is based on the detection and quantitation of a fluorescent reporter. This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. There are two general methods for the quantitative detection of the amplicon: (1) fluorescent probes or (2) DNA-binding agents. The TaqMan probes use the fluorogenic 5' exonuclease activity of Taq polymerase to measure the amount of target sequences in template samples. TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the probe. This ends the activity of the quencher and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye (note that primers are not labeled). The cheaper alternative is the double-stranded DNA binding dye chemistry, which quantitates the amplicon production (including non-specific amplification and primer-dimer complex) by the use of a non-sequence specific fluorescent intercalating agent (SYBR-green I or ethidium bromide). SYBR Green I is a minor groove binding dye. It does not bind to ssDNA.

The threshold cycle or the C_T value is when the system begins to detect the increase in the signal associated with an exponential growth of PCR product during the log-linear phase. This phase provides the most useful information about the reaction (certainly more important than the end point). The important parameter for quantitation is the C_T. The higher the initial amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower the C_T value.

Primers were designed for the promoter regions of GSTP1, androgen receptor (AR), CD44, and β-actin genes. Varying quantities of human placental DNA (80ng, 20ng, 5ng, 1.25ng) was used as template in triplicate for the real time PCR. The amplification for the GSTP1 and AR promoters involved use of a TaqMan probe while the CD44 and β-actin promoters were amplified using SYBR Green.
The threshold values (C_T) were plotted against the log ng of input DNA and the regression equation so generated can be used to quantitate the input quantity of DNA from an unknown sample by using the threshold value.

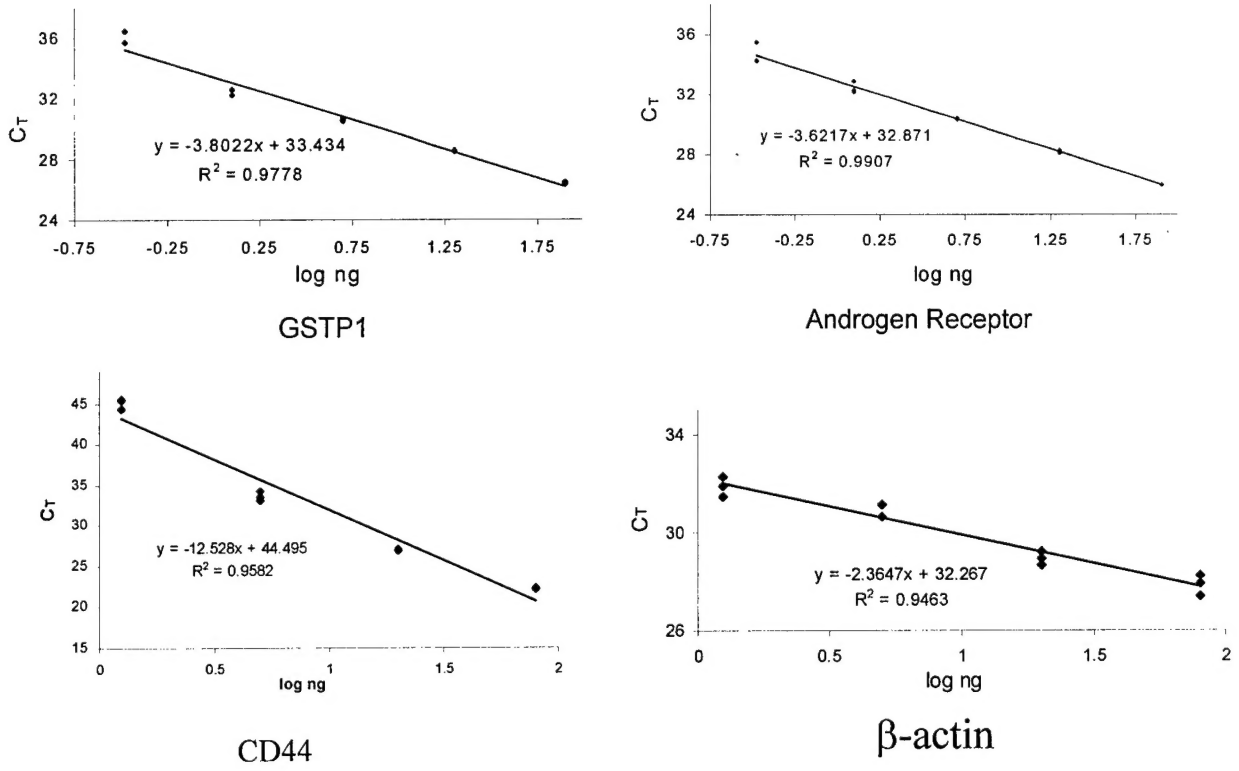

Figure 1. Real time PCR for candidate genes - standard plot. Varying quantities of human placental DNA (80ng, 20ng, 5ng, 1.25ng) was used as template in triplicate for the real time PCR. The threshold values (C_T) were plotted against the log ng of input DNA and the regression equation so generated can be used to quantitate the input quantity of DNA from an unknown sample by using the threshold value.

B. To determine optimal fixation and sonication conditions for the prostate cancer cell lines (months 3-6).

We initially treated 75 cm^2 tissue culture flasks of confluent LNCaP cells with 1% formaldehyde for 30 and 60 minutes. The cell lysate was sonicated using 550 Sonic dismembrator (Fisher Scientific) at 35% power for a variable number of 15 second pulses. As shown in Figure 2, Panel A, even 9 pulses of 15 seconds each failed to sonicate LNCaP chromatin adequately. Decreasing the incubation time to 10 minutes and using 7 pulses of 10 seconds each at 35% power, sonication was only slightly better (Figure 2, Panel B). However, when we decreased the concentration of formaldehyde, we were able to sonicate chromatin adequately for chromatin immunoprecipitation assays (Figure 2, Panel C).

Similarly fixation with 0.5% formaldehyde was found to be optimal for DU 145 and PC 3 cells as well (Figure 3).

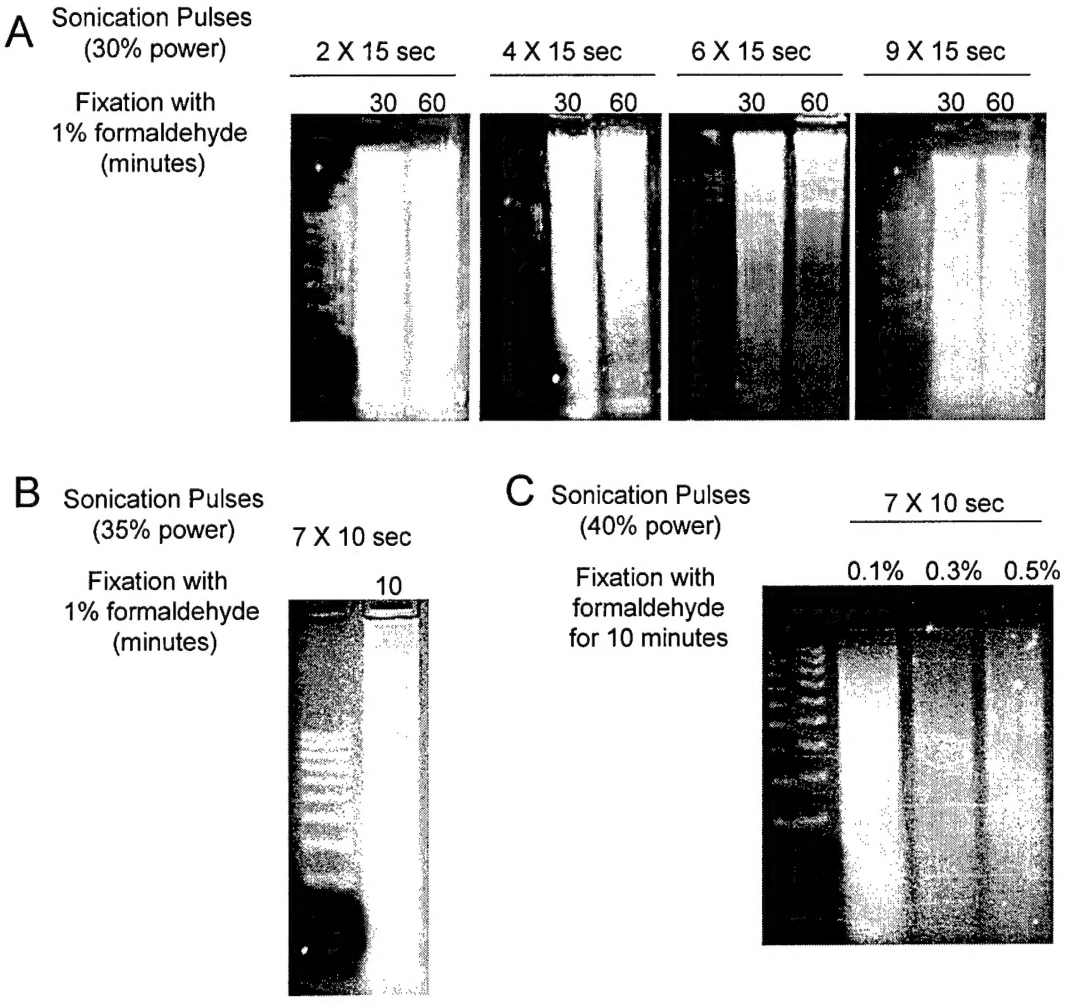

Figure 2. Determination of the optimal fixation and sonication conditions for chromatin immunoprecipitation assay for LNCaP cells. The tissue culture flasks of confluent LNCaP cells were treated with varying concentration of formaldehyde for variable time periods. After lysis with SDS buffer chromatin was sonicated. An aliquot of sonicated chromatin was treated with proteinase K and DNA isolated, and examined on an ethidium stained agarose gel. The size standards shown is a 100bp ladder.

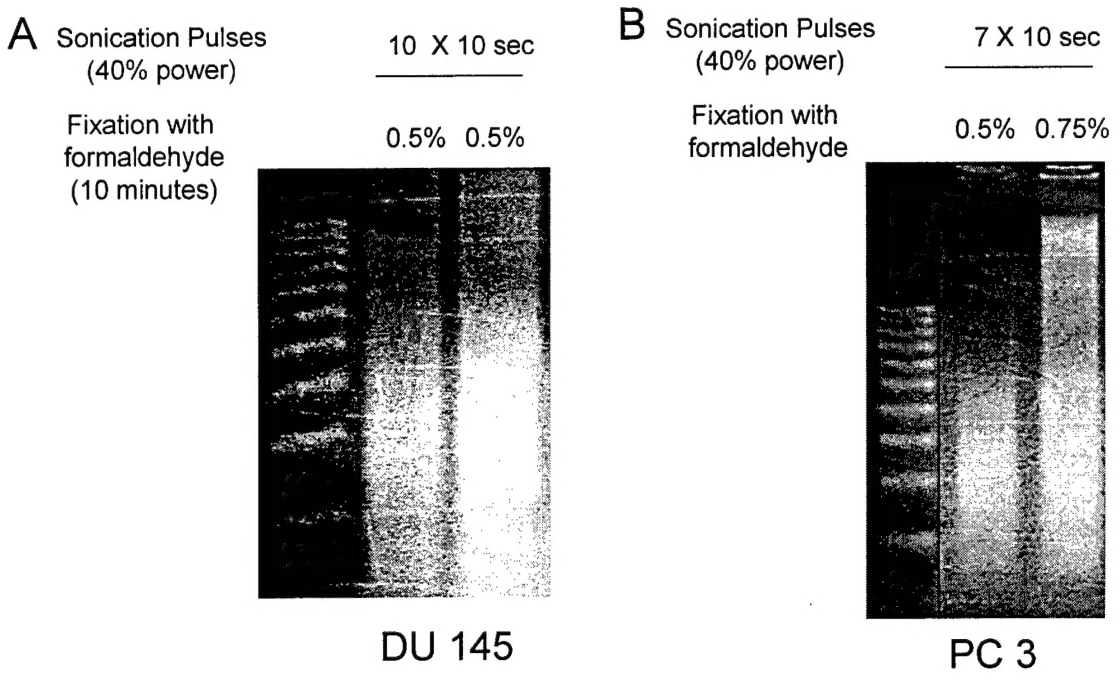

Figure 3. Determination of the optimal fixation and sonication conditions for chromatin immunoprecipitation assay for DU145 and PC3 cells. The tissue culture flasks of confluent cells were treated with formaldehyde. After lysis with SDS buffer chromatin was sonicated. An aliquot of sonicated chromatin was treated with proteinase K and DNA isolated, and examined on an ethidium stained agarose gel. The size standards shown is a 100bp ladder.

3. Perform Chromatin Immunoprecipitation (ChIP) Assays (months 7 - 12)

Chromatin immunoprecipitation assays were carried out with a kit from Upstate Biotechnology using the manufacturer's protocol and reagents except that the reactions were scaled down ten-fold. Briefly, 2 x 10^7 cells were incubated in 0.5% formaldehyde for ten minutes to crosslink bound proteins, washed, lysed in SDS lysis buffer and sonicated to 100-500 bp lengths. Ten µl chromatin was mixed with 90µl dilution buffer and precleared with Protein A agarose, and then the chromatin was incubated with antibody overnight at 4°C. Thirty µl of Protein A agarose beads was added and the chromatin was immunoprecipitated 2 hours at 4^0C. The supernatant (unbound chromatin) and beads (bound chromatin) were separated. The beads were washed five times with the buffers provided and then the chromatin was eluted twice in 1%SDS in 0.1M $NaHCO_3$. Both bound and unbound chromatin fractions were de-crosslinked by the addition of 5M NaCl and incubation at 65°C for at least 4 hours. Proteins were digested by Proteinase K and then chromatin was extracted with phenol/chloroform. DNA was ethanol precipitated and dissolved in 100µl of water. The experiment was repeated on three separate occasions.

Quantitative Real-time PCR The real time PCR was performed in triplicate using 10µl of bound DNA and 5µl of unbound DNA template obtained after chromatin immunoprecipiation, the TaqMan or SYBR Green PCR Master Mix (Applied Biosystems), and primers only (SYBR Green) or primers and probe (TaqMan). When

SYBR Green was used, a dissociation curve was created using software from Applied Biosystems to confirm the presence of a single PCR product. Relative quantitation of template DNA was performed as described in the User Bulletin #2, ABI Prism 7700 Sequence Detection System (Applied Biosystems).

We have compared the levels of histones H3 and H4 acetylation at the methylated and silenced *GSTP1* promoter with an unmethylated and actively expressed AR promoter in LNCaP cells using a chromatin immunoprecipitation assay. Formaldehyde cross-linked chromatin was immunoprecipitated with antibodies against acetylated histones H3 and H4. The unbound and antibody-bound DNA was analyzed for the *GSTP1* and *AR* gene promoters using a quantitative real time PCR approach and the ratio of bound to unbound DNA calculated (Figure 4). As compared to the *AR* promoter, the *GSTP1* promoter was enriched in deacetylated histones H3 ($t = -3.99$, $p < 0.001$) and H4 ($t = -4.076$, $p < 0.001$) (Figure 4). These results are consistent with the recruitment of histone deacetylase containing complexes by methylated DNA, resulting in a localized deacetylation.

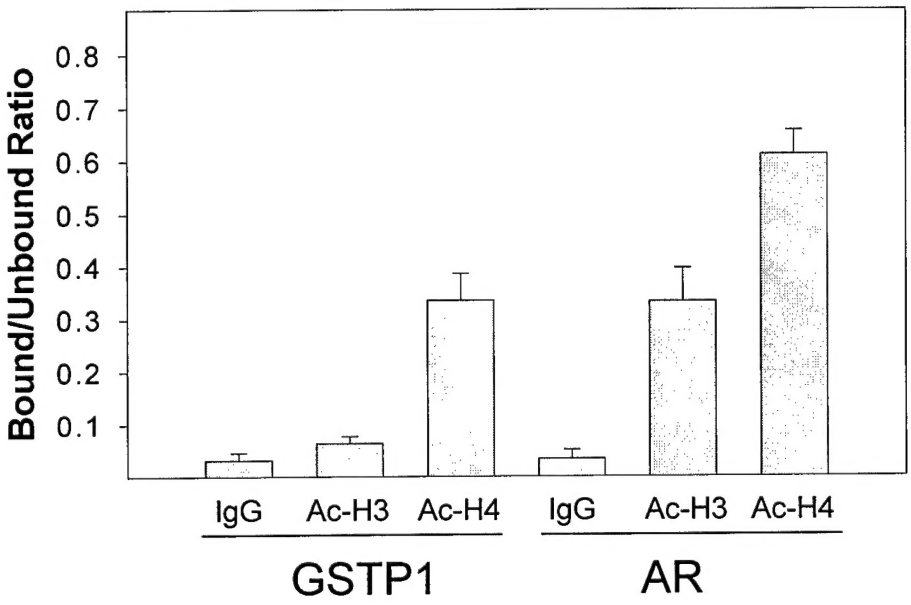

Figure 4. Chromatin Immunoprecipitation (ChIP) Assays. ChIP assays were performed using non-specific immunoglobulin (IgG) and acetylated histone H3 (Ac-H3) and acetylated histone H4 (ac-H4) antibodies. At least 8 separate PCR reactions from 3 different experiments were performed. Error bars indicate the standard error of mean. The *AR* promoter is hyperacetylated at both histones H3 and H4 as compared to the *GSTP1* promoter.

Key Research Accomplishments

- We have determined the linear PCR conditions for the proposed genes.
- We have determined the optimal fixation and sonication conditions for the prostate cancer cell lines.
- We have performed chromatin immunoprecipitation assay for the GSTP1 and AR promoters in LNCaP prostate cancer cell lines. As compared to the *AR* promoter, the *GSTP1* promoter was enriched in deacetylated histones H3 and H4. These results are consistent with the recruitment of histone deacetylase containing complexes by methylated DNA, resulting in a localized deacetylation.

Reportable Outcomes

None

Conclusions

We are making good progress on the present project. The progress report covers the period April 1, 2002 - March 31, 2003. However, the distribution of funds was stopped effective October 1, 2002 pending the transfer of Prinicipal Investigator to University of Miami. We have received 6 months of funding for this project so far. The above results indicate that we have met the goals that were set out in the statement of work.

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APPENDICES
None